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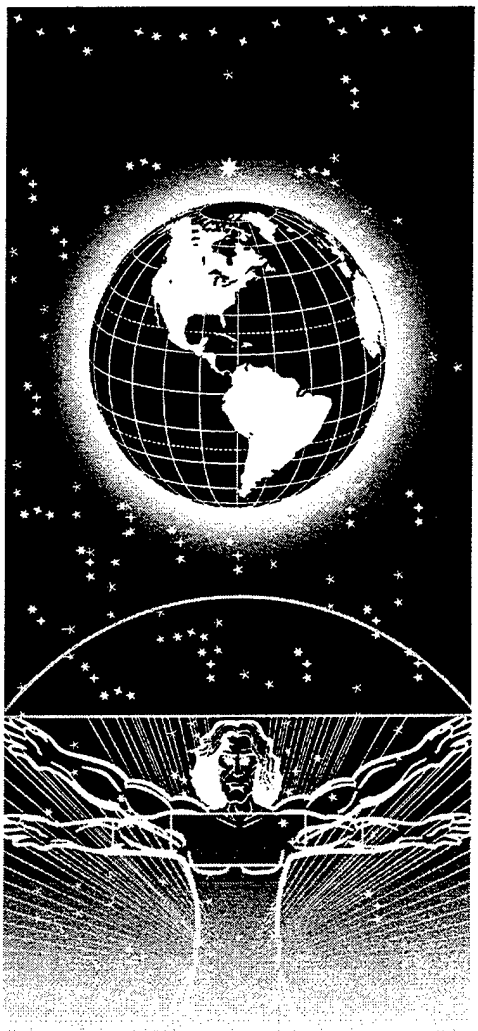

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

NONGEL DETECTION OF PCR AMPLICONS DIAGNOSTIC OF *E. COLI*, 0157:H7: POTENTIAL FOR USE IN FIELD CONDITIONS

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The Office of Public Affairs has reviewed this technical report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

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13. ABSTRACT (Maximum 200 words)

The Polymerase Chain Reaction (PCR) has provided powerful new strategies for the detection and identification of bacterial pathogens. We routinely use PCR-based methods to detect, identify, and study *E. coli* 0157:H7 in our laboratory. Generally, after PCR, agarose gel electrophoresis is performed to visualize and characterize amplicons. However, amplicons of similar sizes cannot always be differentiated by electrophoretic methods, and spurious amplicons may occasionally be generated under multiplex PCR conditions. Southern hybridization methods can be employed to clear up ambiguities, but such measures can be laborious and time-consuming. We have used a rapid paper chromatography-based method, purchased in kit form (GeneComb™, BIO RAD), to rapidly and easily analyze PCR-generated products. The method is based upon chromogenic detection of amplicons generated through the use of biotinylated primers, coupled with a brief (30 min.) chromatography step. This method allows complete clinical analysis of PCR amplicons in as little as one hour, providing positive identification of *E. coli* 0157:H7. Multiple amplicons can be definitively detected depending upon the primers employed, the PCR conditions, and design of the chromatography step. We have used the method to easily, rapidly, and positively identify the 90-100 kb virulence plasmid of *E. coli* 0157:H7, and to successfully differentiate between two similar-sized amplicons of the Shigella-like toxins (SLT I and SLT II) of 0157:H7 strains.

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Contents

Introduction

Escherichia coli O157:H7 is an important pathogen which causes hemorrhagic colitis and hemolytic uremia syndrome (HUS) (4, 6). Disease outbreaks due to *E. coli* O157:H7 have been reported in nursing homes (5) and day care centers (6), arising from contaminated meat, water and even bottled juice. *E. coli* O157:H7 isolates typically possess a 90-100 kb plasmid that encodes pathogenic functions, and also express two distinct phage-encoded Shigella-like toxins (SLT I and SLT II). Various authors have demonstrated the diagnostic value of probes specific for these molecules (2, 3).

One prime consideration for the routine application of PCR-based detection methods for detection of *E. coli* O157:H7 is the ability of personnel easily to carry out the various steps. These steps can be conceptually divided into three phases: 1) sample preparation, 2) amplification and 3) detection of the amplicon. Detection of the amplified DNA, is usually accomplished by agarose gel electrophoresis and subsequent viewing of the ethidium bromide-stained PCR product. This step has remained largely unchanged since the advent of PCR, even though broad advances in simplifications have been made in the first two phases. In our experience, clinically trained personnel can often experience difficulty in preparing and casting the gel, loading the samples in the gel, performing the actual electrophoresis, staining the gel, visualizing and photographing the product, and interpreting the result. Moreover, electrophoresis may not even be possible under some field conditions.

We have examined use of a chromatography-based method (Universal GeneComb™, BioRad, Hercules CA) to detect PCR amplicons specific for either: 1) the plasmid of *E. coli* O157:H7, 2) the Shigella-like toxin genes of *E. coli* O157:H7 (SLT I and SLT II). The goal was to determine the efficacy of chromatography-based methods to detect biotinylated PCR amplicons as well as their potential usefulness in situations where laboratory personnel might have limited access to equipment and facilities. A total of 57 bacterial strains were examined, including 22 *E. coli* O157:H7 strains and 35 other *E. coli* strains or *Shigella* spp.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacteria used in this study are listed in Table 1. A total of 40 bacterial strains were examined, including 19 *E. coli* O157:H7 strains. The O157:H7 bacteria included clinical isolates from the Centers for Disease Control and Prevention (Atlanta GA), the Alabama Department of Health (Montgomery AL), the Texas Department of Health (Austin TX), Brooks Air Force Base Armstrong Laboratory (San Antonio TX), and bovine isolates from the Auburn University School of Veterinary Medicine (Auburn AL). Other clinical isolates of *E. coli* representing a variety of other serotypes, as well as clinical isolates of various *Shigella* spp., were included in the study for comparison. Environmental isolates of *E. coli* were from a variety of sources. Strains were characterized biochemically by the following tests: VITEK® GNI (bioMerieux Vitek, Inc. Hazelwood MO), Premier EHEC Enzyme Immunoassay Test (Meridian Diagnostics,

Inc., Cincinnati OH), Oxoid *E. coli* O157 kit (Unipath Limited, Hampshire, England), MacConkey agar or MacConkey agar with sorbitol (Remel, Lenexa KS). Additionally, the presence of the H7 antigen was assayed by the method of Farmer and Davis (1). All bacteria were routinely cultured at 35° C on brain heart infusion agar (BHI) or Luria Bertoni (LB) agar (Remel). Antibiotic sensitivity was determined by the VITEK® GNS card (bioMerieux Vitek, Inc.). Additionally, to determine sensitivity to streptomycin, bacteria were streaked onto LB plates supplemented with streptomycin sulfate (Sigma Chemical Co., St. Louis MO) at 50 µg/ml. Bacterial transformants were selected on LB agar supplemented with ampicillin (Sigma

Nucleic Acid Manipulations

Genomic DNA was extracted from *Escherichia* and *Shigella* strains for PCR as described by Fratamico et al. (2). Briefly, a single colony from an overnight culture was resuspended in 200 ul of lysis buffer (0.5% Triton X-100, 20 mM Tris pH 8.0, 2 mM EDTA) and boiled for 10 minutes to lyse the cells. All oligonucleotides were prepared by Midland Certified Reagent Co. (Midland TX). Where required, oligonucleotides were biotinylated by addition of a 5' biotin-labeled T residue.

Two primers (MK1 and MK2) were employed to amplify the SLT sequences that are diagnostic of O157:H7 isolates. The sequence of MK1 is 5'TTTACGATAGACTTCTCGAC3' and that of MK2 is 5'CACATATAAATTATTTCGCTC3'. Two different oligonucleotide probes based upon the report of Karch and Meyer (3) were employed to probe for each of the two different SLT amplicons. The oligonucleotide, MKP1, 5'GATAGTGGCTCAGGGGATAA3', was synthesized to detect SLT I. To detect SLT II sequences the probe MKP2, 5'AACCACACCCACGGCAGTTA3' was constructed.

Primers, MS1F and MS1R, 5'ACGATGTGGTTTATTCTGGA3' and 5'CTTCAGTCACCATACATAT3', respectively, were used for specific amplification of a 166 bp fragment the 90 kbp plasmid that is found in *E. coli* O157:H7 strains (2). The oligonucleotide probe, MSFPP, 5'CCGTATCTTATAATAAGACG 3', was prepared to detect the 166 bp plasmid amplicon (2).

Five to ten ul of colony lysate along with 50 pmol of each primer was added to an amplification volume of 100 ul. A multiplex PCR protocol (2) was employed to amplify the plasmid sequence and both SLT sequences. The reaction mix was 2.0 $MgCl_2$, 20 mM Tris (pH 8.0), 50 mM KCl, 0.001% gelatin, 200 uM for each of the four dNTPs (800 uM total), with amplification as follows. An initial denaturation of 94° C for 5 minutes was followed by 35 cycles of denaturation (1 min., 94° C), annealing (3 min., 48° C) and extension (4 min., 72° C), followed by a single period of extension for 5 minutes at 72° C.

Following amplification, 10 ul of the PCR reaction was analyzed by agarose (1.6%) gel electrophoresis and subsequent visualization with ethidium bromide. Additionally, another 10 ul was analyzed by means of the Universal GeneComb™ (BioRad, Hercules CA). The GeneComb has eight nitrocellulose teeth, one of which is reserved for a control reaction. On each tooth, one or two probe spots may be utilized for evaluation of the PCR products. In all of our tests, two

probe spots per tooth were utilized. Ten pmol of oligonucleotide probe was deposited onto the teeth in accordance with the manufacturer's instructions. The probe was covalently affixed to the nitrocellulose by a three minute exposure on the same ultraviolet transilluminator that was utilized for visualization of ethidium bromide stained amplicons. Chromatography of the amplicons and color development of the duplex molecules were as described by the manufacturer. A purple spot on the comb, in the area of the probe was recorded as a positive result, the lack of a spot in the expected area was recorded as a negative result.

To examine the flexibility of the comb, a simple experiment was designed to determine if a single probe spot could provide information about multiple amplicons. Thus, in some determinations, the probe for SLT I (MKP1) and the probe for SLT II (MKP2) were mixed together in equal portions and both were added to a single probe spot on a tooth.

Results

Electrophoresis Results

Two bands of the expected sizes (166 bp and 224/227 bp) were produced from the DNA isolated from the O157:H7 strains. There were two exceptions. One O157:H7 strain obtained from the American Type Culture Collection does not produce either SLT I or SLT II (A57), thus as expected, only a 166 bp amplicon was obtained from this strain. Another O157:H7 strain that had been cured of the large plasmid yielded only a band of 224/227 bp (A59). None of the non-O157:H7 *E. coli* strains nor any of the *Shigella* isolates yielded amplicons in the 166 or 224/227 bp range. But some of these non-EHEC *E. coli* strains did yield amplicons in the 300-400 bp range.

Universal GeneComb™ Results

The GeneComb successfully detected all of the amplicons that were observed via agarose gel electrophoresis. The simplest design employed two assay spots per comb tooth. The upper spot utilized two probes (MKP1 and MKP2) and successfully detected amplicons of either SLT I or SLT II, or both. The lower assay spot detected the 166 bp plasmid amplicon (MSFPP). A positive response in either or both positions was diagnostic of *E. coli* O157:H7. None of the other *E. coli* or *Shigella* strains tested positive. Although some nonpathogenic stains of *E. coli* (e.g. DH5) consistently yielded spurious bands in the PCR reaction, these were not observed when the PCR product was assayed by the GeneComb.

The SLT I and SLT II amplicons are similar in size and indistinguishable via agarose gel electrophoresis. Therefore, it was of interest to know if the GeneComb could differentiate between these two amplicons. In this test the upper assay spot contained only the probe for SLT I (MKPI) whereas the lower assay spot contained only probe for SLT II (MKP2). Table 1 shows that the GeneComb efficiently differentiated between the two amplicons, successfully revealing those strains that produce one, both or neither of the toxins, including two *E. coli* C600 strains transduced with one or the other of the toxigenic pages (7).

The GeneComb and kit proved easy to use and the only special equipment required was a 37° C incubator. But we did observe that the assays described here could be performed at room temperature and still provide adequate results.

Discussion

Three to four hours time was required in order to detect PCR amplicons by agarose electrophoresis, whereas less than an hour was required to detect amplicons by the GeneComb method. Furthermore, if combs were preloaded with the desired probes, assay time could be reduced to 40 min.

There were various advantages in the use of the GeneComb as compared to electrophoretic procedures as far as detection of amplicons was concerned. First, the GeneComb easily differentiated between the two different SLT amplicons. At 227 and 224 bp in size, these amplicons for SLT I and SLT II were indistinguishable via electrophoresis, but were easily and successfully differentiated by the GeneComb test depending upon the layout of the probes on the comb teeth. To differentiate between the two SLT amplicons, probes specific to each were deposited on the comb in different assay spots. When SLT I and SLT II probes were placed in the same assay spot, amplification of either one or both toxin genes was recorded as a positive result demonstrating that multiple probes can be incorporated into each spot. Such an approach depends on an experimental design wherein detection of one or both DNA sequences (such as the SLTI and SLTII sequences) provides an acceptable clinical answer.

Another advantage with the GeneComb was that spurious amplicons were not visualized. These spurious amplicons were generated by the multiplex PCR procedures utilized here, and these extra bands were easily detected by agarose electrophoresis, but not by the GeneComb procedure. These extra bands could cause confusion during result interpretation if they were interpreted as positive results, as might be the case by newly trained personnel.

Additionally, the GeneComb was able to detect very small amounts of amplicon, less than could be detected visually. Generally, one tenth (10 ul) of the PCR reaction was used in either electrophoretic or GeneComb tests and this amount proved more than sufficient for detection purposes. However, when the PCR products were diluted 100-fold, only the GeneComb consistently detected the amplicons. Biotinylation of the primers had no effect on the PCR results, and amplicons were generated successfully through the use of nonbiotinylated or biotinylated primers.

In summary, these results taken together support several conclusions. The GeneComb is simpler and more rapidly used (< 1 hr vs. 3-4 hr) than electrophoretic methods and it is as reliable as electrophoretic methods and, additionally, it eliminates "noise" due to generation of unwanted or spurious bands. The GeneComb can also easily distinguish between amplicons of similar size but different sequence, and it is more sensitive than agarose electrophoresis, consistently being able to detect 100-fold less than the standard assay amount. These observations suggest that the GeneComb may be useful in situations where electrophoresis may prove difficult to perform, where amplicons may be of similar sizes, where little amplicon may

be generated, or where personnel untrained in molecular methods may be required to interpret the results of PCR tests.

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Table 1. Electrophoretic and GeneComb Results

Representative Results from the *E. coli* and *Shigella* Isolates Tested.

	Reference or Source	Identifying Info.	Electrophoresis Result		Universal	Gene Comb Result
			plasmid DNA,(a 166 bp amplicon)	SLT I or SLT II, (a 224/227 bp amplicon)	plasmid specific amplicon	SLT I or SLT II[a] specific amplicon
A11	CDC 8958	*E. coli* O157:H7	+	+	+	+
A13	(7)	*E. coli* C600 expressing SLT I (phage 933J)	-	+	-	SLT I only
A14	(7)	*E. coli* C600 expressing SLT II (phage 933W)	-	+	-	SLT II only
A21	Bovine isolate, Auburn University	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A23	ATCC 25922	*E. coli*, non O157:H7	-	-	-	-
A24	environmental isolate, this study	*E. coli*, non O157:H7	-	-	-	-
A44	clinical isolate, this study	*E. coli*, non O157:H7	-	-	-	-
A45	clinical isolate, this study	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A46	clinical isolate, this study	*E. coli*, non O157:H7	+	+	+	+
A47	ATCC #43894	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A48	ATCC #33849	*E. coli* DH1	-	-	-	-
A49	ATCC #35150	*E. coli* O157:H7	+	+	+	+
A50	ATCC #43895	*E. coli* O157:H7	+	+	+	+
A51	ATCC #33694	*E. coli* HB101	-	-	-	-
A57	ATCC #43888	*E. coli* O157, with neither toxin	+	-	+	-
A58	ATCC #43890	*E. coli* O157:H7	+	+	+	SLT I only
A59	ATCC #43894	*E. coli* O157:H7 cured of 60 Mda plasmid	-	+	-	SLT I and SLT II
A60	BE4-32, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A61	BE5-190, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A62	BE5-124, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A64	BE6-266, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A65	BE5-412, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A71	BE5-791, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A78	BE5-1198, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A79	BE4-1207, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A86	BE3-1404, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A98	BE3-1676 Texas Dept. of Health	*E. coli* O157:H7	+	+	+	+
A112	BE4-1269, Texas Dept. of Health	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A123	ATCC #53868	*E. coli* DH5	-	-	-	-
A124	clinical isolate, this study	*E. coli* O157:H7	+	+	+	SLT I and SLT II
A125	clinical isolate, this study	*E. coli* O126	-	-	-	-
A126	clinical isolate, this study	*E. coli* O111	-	-	-	-
A127	clinical isolate, this study	*E. coli* O55	-	-	-	-
A128		*Shigella boydii*	-	-	-	-
A129		*S. flexneri*	-	-	-	-
A130	clinical isolate, this study	*E. coli* O126	-	-	-	-
A131	CDC 2167-57	*E. coli* O28	-	-	-	-
A132	CDC 1001-59	*E. coli* O112	-	-	-	-
A133	CDC 1871-56	*E. coli* O128	-	-	-	-
A134	CDC 2160-53	*E. coli* O127	-	-	-	-

Note: A plus sign indicates either SLT I or SLT II, or both, was detected, if a distinction between the two was made the results are clearly indicated. A minus sign indicates that neither SLT I nor SLT II was detected.

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